

PROJECT ADMINISTRATION DATA SHEET

ORIGINAL



REVISION NO. _____

Project No. G-33-687 R5927-1A0GTRC~~XXXX~~DATE 5 / 5 / 86Project Director: Dr. James C. PowersSchool/~~XXX~~ ChemistrySponsor: DHHS/PHS/NIH/NHLBIType Agreement: Grant No. 5 R01 HL34035-02Award Period: From 4/1/86 To 3/31/87 (Performance) 3/31/87 (Reports)

Sponsor Amount:

This ChangeTotal to DateEstimated: \$ _____ \$ 105,337.00Funded: \$ _____ \$ 105,337.00Cost Sharing Amount: \$ 5,544.00 Cost Sharing No: G-33-320Title: Inhibitors for Blood Coagulation ProteasesADMINISTRATIVE DATAOCA Contact E. Faith Gleason X4820

1) Sponsor Technical Contact:

2) Sponsor Admin/Contractual Matters:

Dr. Diane LucasJon Carow (301) 496-7255Division of Blood Diseases and ResourcesGrants Operation BranchNational Heart, Lung and Blood InstituteDivision of Extramural AffairsFederal Bldg., Room 5A12National Heart, Lung and Blood InstituteBethesda, Maryland 20892National Institutes of HealthBethesda, Maryland 20892

Defense Priority Rating: _____

Military Security Classification: _____

(or) Company/Industrial Proprietary: _____

RESTRICTIONSSee Attached NIH Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval — Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of \$500 or 125% of approved proposal budget category.

Equipment: Title vests with GITCOMMENTS:02 year of continuing grant.Previous Project No. was G-33-606.COPIES TO:SPONSOR'S I. D. NO.Project Director
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SPONSORED PROJECT TERMINATION/CLOSEOUT SHEET

Date 6-4-87

Project No. G-33-687

School XXX Chemistry

Includes Subproject No.(s) N/A

Project Director(s) James C. Powers

GTRC XXX

Sponsor DHHS/PHS/NIH/NHLBI

Title Inhibitors for Blood Coagulation Proteases

Effective Completion Date: 3/31/87 (Performance) 3/31/87 (Reports)

Grant/Contract Closeout Actions Remaining:

- ☐ None
- ☒ Final Invoice or Final Fiscal Report
- ☐ Closing Documents
- ☐ Final Report of Inventions
- ☐ Govt. Property Inventory & Related Certificate
- ☐ Classified Material Certificate
- ☐ Other _____

Continues Project No. G-33-606

Continued by Project No. G-33-619

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 Other Duane H.
Angela DuBose
Russ Embry

SECTION IV PROGRESS REPORT SUMMARY		GRANT NUMBER HL34035-03	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR Powers, James C.		PERIOD COVERED BY THIS REPORT	
NAME OF ORGANIZATION Georgia Tech Research Institute		FROM 4/1/86	THROUGH 2/19/87
TITLE (Repeat title shown in item 1 on first page) Inhibitors For Blood Coagulation Proteases			
(SEE INSTRUCTIONS)			

Publications

"Mechanism-Based Isocoumarin Inhibitors for Trypsin-Like Serine Proteases Involved in Blood Coagulation," C-M. Kam, J.C. Copher, and J.C. Powers (1987) J. Am. Chem. Soc. 109, submitted as a Communication to the Editor.

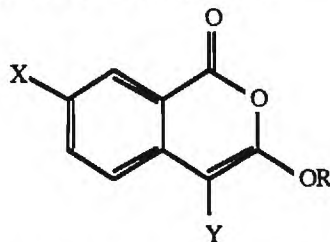
Scientific Goals

The goals of this research are the design, synthesis, and testing of synthetic inhibitors for the serine proteases involved in the blood coagulation pathway. Both novel transition state inhibitors and mechanism-based (suicide) inhibitors will be evaluated with the purpose of obtaining compounds which will selectively inhibit only one or a few of the activated coagulation factors, and compounds which will be general inhibitors of most of the activated coagulation factors.

This research should lead to a better understanding of the active site structures of the blood coagulation serine proteases, both individually and in comparison to other trypsin-like serine proteases; may produce clinically useful drugs; should stimulate medicinal chemists in pharmaceutical companies to synthesize more enzyme targeted antithrombotic agents; and will provide new tools for the *in vivo* and *in vitro* study of the role of the individual coagulation factors in blood coagulation.

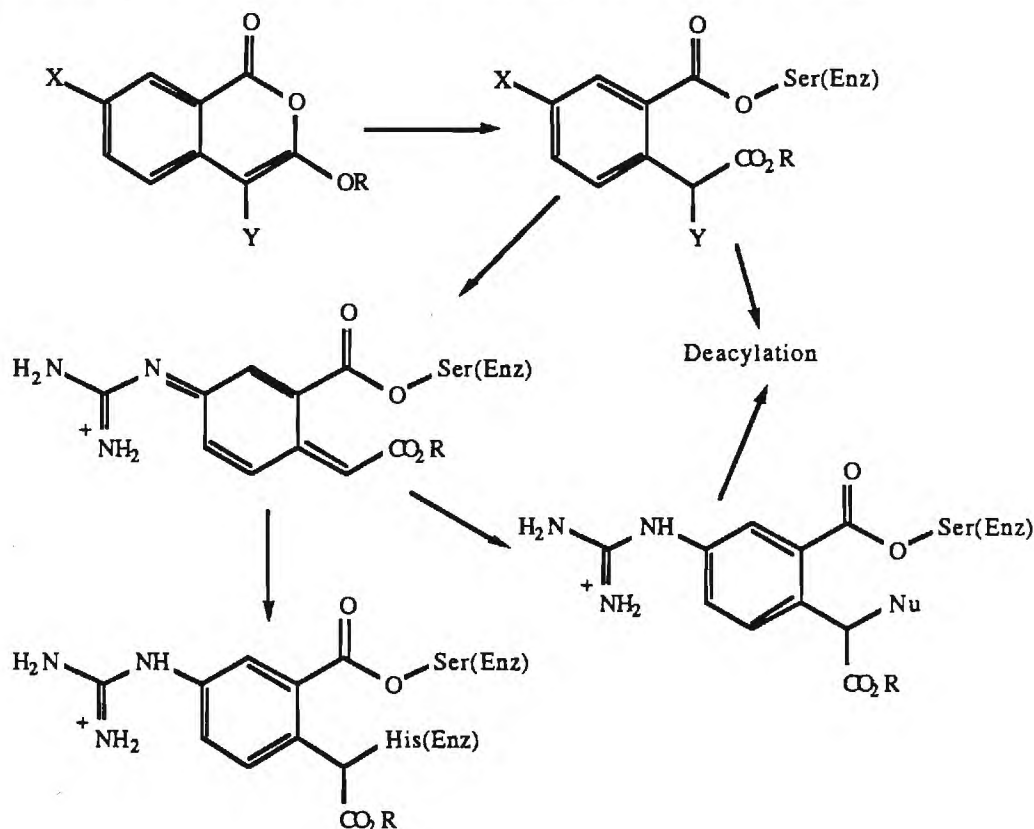
Progress Report

Mechanism-Based Inhibitors. We have made substantial progress in the synthesis of isocoumarin mechanism-based inhibitors for coagulation proteases and related trypsin-like enzymes.



The structures of some of the inhibitors are **1**, R = $\text{CH}_2\text{CH}_2\text{SC}(=\text{NH}_2^+)\text{NH}_2$, X = H, Y = Cl; **2**, R = $\text{CH}_2\text{CH}_2\text{CH}_2\text{SC}(=\text{NH}_2^+)\text{NH}_2$, X = H, Y = Cl; **3**, R = $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_3^+$, X = H, Y = H; **4**, R = $\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_3^+$, X = H, Y = Cl; **5**, R = CH_3 , X = $\text{NHC}(=\text{NH}_2^+)\text{NH}_2$, Y = H; **6**, R = CH_3 , X = $\text{NHC}(=\text{NH}_2^+)\text{NH}_2$, Y = Cl.

Incubation of the the isocoumarins with several coagulation enzymes and trypsin resulted in a time-dependent loss of enzymatic activity (Table I). The most potent inhibitor toward thrombin, trypsin and porcine pancreatic kallikrein was 4-chloro-7-guanidino-3-methoxyisocoumarin **6**. Human protein D, human leukocyte elastase, human leukocyte cathepsin G, porcine pancreatic elastase, chymotrypsin and human plasma plasmin were inactivated with $k_{\text{obs}}/[I]$ values of 122, 6400, 11,000, 900, 7200, 3600 $\text{M}^{-1}\text{s}^{-1}$. The guanidino isocoumarin **6** was quite selective and has its most potent inhibitory activity toward trypsin, thrombin and human plasma kallikrein with $k_{\text{obs}}/[I]$ values of 200,000 - 300,000 $\text{M}^{-1}\text{s}^{-1}$. For all practical purposes, the high inhibition rates mean that stoichiometric amounts of inhibitor will inactivate the enzymes instantaneous. 4-Chloro-3-(2-isothiureidoethoxy)isocoumarin **1** is the most potent inhibitor for plasma kallikrein, human factor XIa and human factor XIIa.



The inhibition mechanism involves reaction of the 3-alkoxy-4-chloroisocoumarins **1**, **2**, **3**, **4**, and **5** with the active site serine residue of serine proteases to form acyl enzymes (top right) which deacylate slowly at varying rates or quickly upon the addition of hydroxylamine. If the 7-substituent on the isocoumarin ring is a guanidino (**6**) or amino group then further reaction through a quinone imine methide (center) can take place to give an irreversibly inactivated enzyme by reactions with an an active site nucleophile such as His-57 (bottom left). The quinone imine methide intermediate could also react with a solvent nucleophile to give an acyl enzyme (middle right) which can be reactivated by hydroxylamine.

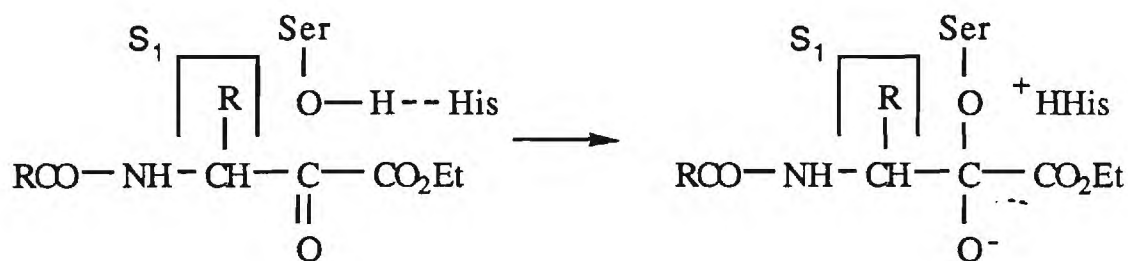
Anticoagulant Activity. The 7-guanidino isocoumarin **6** is an effective anticoagulant in human plasma. The prothrombin time was prolonged from 12 sec (first appearance of clotting) to 2.8 min in the presence of 33 μM **6**. The general serine protease inhibitor 3,4-dichloroisocoumarin prolonged the prothrombin time to 2 min at 330 μM . The isocoumarin **6** decomposed quite rapidly in human plasma ($t_{1/2} = 5$ min.) and preincubation of the inhibitor in plasma for 3 min resulted only in a 80 % increase in prothrombin time.

One specific aim for next year is the testing of the inhibitors with additional coagulation enzymes such as protein C and factor VIIa, and with other trypsin-like enzymes. Another goal is the synthesis of other isocoumarin derivatives with increased selectivity toward specific coagulation proteases. The PI has begun a program of modeling the inhibitors with the active sites of trypsin and porcine pancreatic kallikrein, the only two trypsin-like enzymes whose x-ray structures are known. We hope to learn how the inhibitors are interacting with the enzymes in order to introduce structural features which will lead both to increased selectivity and reactivity. Prof. Bud Suddath, an x-ray crystallographer at Georgia Tech is planning a crystal structure of one of our inhibitors bound to trypsin. This will yield important structural information on the mode of binding of the inhibitors. Finally, we plan to contact investigators who are carrying out animal studies of coagulation in order to have some of our inhibitors tested in animals.

Transition State Analog Inhibitors. The first class of transition state inhibitors which we have been synthesizing are α -ketoesters derived from Lys. These are prepared from the corresponding blocked amino acid or peptide. At present the following compounds have been prepared and purified. The synthesis and purification of these compounds turned out to be much more difficult than expected and it takes considerable effort to prepare each structure.

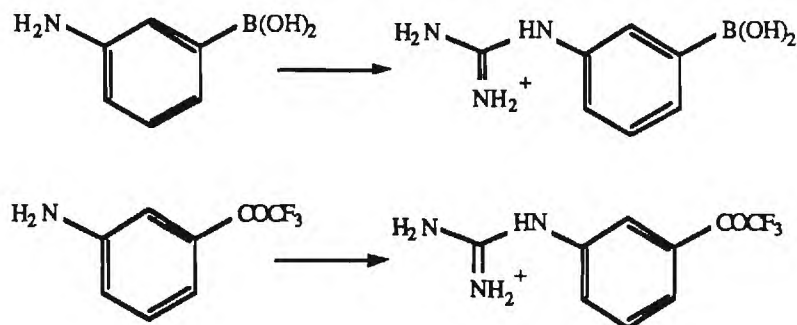
Bz-Lys-CO₂Et
Gly-Lys-CO₂Et
Pro-Lys-CO₂Et
Phe-Lys-CO₂Et
Ala-Lys-CO₂Et
Leu-Ala-Lys-CO₂Et

We expect that these compounds will interact with the active sites of trypsin-like serine proteases to form tetrahedral intermediates as shown below. Only preliminary kinetic studies have been carried out with the α -ketoesters up to the present. We have shown that the first three derivatives inhibit trypsin with kinetic constants in the μM range. None have yet been tested with any of the coagulation factors or thrombin. In one preliminary experiment with the Bz-Lys-CO₂Et, we found some inhibition of clotting in human plasma.



Our first priority for the next year, indeed the next few weeks, is to carry out kinetic studies with the α -ketoesters shown above. First we plan to obtain inhibition constants with the purified enzymes and then proceed to clotting assays with the most potent inhibitors. We plan to evaluate the results at that point and make a decision whether to proceed with the synthesis of longer peptidyl α -ketoesters derivatives or to move to inhibitors with other types of functional groups.

Two other classes of inhibitors which we are planning to synthesize are boronic acids and trifluoromethyl ketones (shown below). Both of these are transition state inhibitors and should react with the active site serine to form tetrahedral intermediates similar to that shown for the α -ketoesters. We have started with the boronic acids and are now trying to convert the *m*-amino boronic acid into the guanidino compound. The reaction appears to be working, but we have not yet been able to isolate and purify the product. Our goals for next year are to successfully synthesize and test these compounds and related derivatives.



Highlights of Specific Scientific Accomplishments

The guanidino and isothiureidoalkoxy isocoumarins **6** and **1** developed in this study are the most potent inactivators yet reported for the several of the coagulation enzymes studied and **6** is one of the first mechanism-based (or suicide) serine protease inactivator which is active as an anticoagulant in human plasma. Thus we have taken the first step of the road toward the development of a significant class of new antithrombotic drugs.

Table I. Rates of Inhibition of Trypsin-like Serine Proteases by Substituted Isocoumarins^a

Inhibitor	$k_{\text{obs}}/[\text{I}] \text{ (M}^{-1}\text{s}^{-1}\text{)}$						
	bovine thrombin	bovine factor Xa	porcine pancreatic kallikrein	human plasma kallikrein	human factor XIa	human factor XIIa	bovine trypsin
<u>1</u>	4,700	5,600	12,000	280,000 ^b	44,000	39,000	32,000
<u>2</u>	1,400	220	19,000	110,000	47,000	27,000	46,000
<u>3</u>	2.7	NI ^c	4.7	29	28	3.2	1,200
<u>4</u>	350	160	860	1,400	380	190	7,600
<u>5</u>	4,900	460	1,900	13,000	1,400	520	3,300
<u>6</u>	290,000 ^d	3,100	45,000 ^d	240,000 ^d	36,000	20,000	310,000 ^d